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Mechanical Stretch-Induced Changes in Cell Morphology and mRNA Expression of Tendon/Ligament-Associated Genes in Rat Bone-Marrow Mesenchymal Stem Cells

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Abstract: It has been demonstrated that mechanical stimulation plays a vital role in regulating the proliferation and differentiation of stem cells. However, little is known about the effects of mechanical stress on tendon/ligament development from mesenchymal stem cells (MSCs). Here, using a custom-made cell-stretching device, we studied the effects of mechanical stretching on the cell morphology and mRNA expression of several key genes modulating tendon/ligament genesis. We demonstrate that bone-marrow-derived rat MSCs (rMSCs), when subjected to cyclic uniaxial stretching, express obvious detectable mRNAs for tenascin C and scleraxis, a unique maker of tendon/ligament formation, and significantly increased levels of type I collagen and type III collagen mRNAs. The stretched cells also orient at approximately $\sim 65^{\circ}$ with respect to the stretching direction and exhibit a more fibroblast-like morphology. Collectively, these results indicate that mechanical stretching facilitates the directed differentiation of rMSCs into tendon/ligament fibroblasts, which has potential implications for the tissue engineering of bioartificial tendons and ligaments.

Keywords: Mesenchymal stem cells; Mechanical stretching; Cell morphology; Differentiation; Tendon/ligament

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1 Introduction

It is well established that mechanical stimulation is critical for the growth and functioning of living cells. Various effects of mechanical stimulation have been reported at the cellular level. Stress deprivation results in changes in the biochemical and biomechanical properties of cells (1), and excessive strain can increase the inflammatory reaction and may damage tissue, leading to a failure of the healing process (2). Skeletal unloading inhibits the differentiation of rat osteoprogenitor cells (3). Conversely, optimal mechanical stretching induces collagen expression in both skin fibroblasts and pulmonary fibroblasts in vitro, and the cells assume an anabolic phenotype (4, 5). It has also been reported that 0.1 Hz, 9–18% strain induces osteoblast and cementoblast differentiation, accompanied by a decrease in the expression of the epidermal growth factor receptor (EGF-R) in periodontal ligament cells (6). These results indicate that mechanical stimulation plays important roles in cell phenotypes and differentiation.

Tendons and ligaments connect the elements of the musculoskeletal system and are composed of a densely packed collagen-rich connective tissue, which is able to retract with high tensile force. However, inappropriate physical training or excessive repetitive stretching often leads to tendon/ligament overuse injuries. The management of damaged tendons/ligaments is still one of the most challenging problems in orthopedics (7). Interestingly, early controlled mechanical mobilization has had significant favorable effects on tendon healing after complete laceration (8).

Mesenchymal stem cells (MSCs) are an attractive cell source for a wide variety of tissue engineering strategies, because they are a pluripotent pop-

ulation of cells capable of differentiating along multiple mesenchymal cell lineages, including osteoblasts, chondrocytes, and adipocytes, and do not pose the ethical concerns inherent in the use of embryonic or fetal tissue (9). This plasticity offers a promising strategy for improved wound healing in injured tendons/ligaments, and even tissue-engineered tendons/ligaments. Unfortunately, little is known about the selective differentiation of MSCs into tendon/ligament cells. The lack of attention given to tendons/ligaments can be largely attributed to the absence of simple histological staining procedures or specific molecular markers for these tissues. Recently, a new unique early marker for tendon/ligament fibroblasts, scleraxis, was reported, which is a basic helix–loop–helix transcription factor expressed specifically and exclusively in tendons/ligaments (10, 11). This should facilitate better research into the directed differentiation of MSCs into tendon/ligament cells in vitro. Low-dose fibroblast growth factor 2 (FGF-2) increases MSC proliferation and stimulates the mRNA expression of specific extracellular matrix proteins and cytoskeletal elements of tendons/ligaments (12). Altman et al. applied translational and rotational stress to MSCs seeded in a collagen sponge and demonstrated the upregulation of ligament fibroblast-associated gene expression, including the genes for type I collagen (*Col1*), type III collagen (*Col3*), and tenascin C (*Tnc*) (13). Recently, Moreau et al. described the effects of multiple growth factors and medium constituents on gene expression in MSCs on a 2D construct and identified bFGF, epidermal growth factor (EGF), and transforming growth factor β (TGF-β) as effective mediators of fibroblast differentiation (14). Kurpinski et al. compared the responses of smooth muscle cells (SMCs) and MSCs to equiaxial strain, uniaxial strain and mechanical strain in three-dimensional culture (15).

Whereas the aforementioned studies have evaluated the influence of biochemical or mechanical stimulation on the expression of several genes in MSCs, the influence of mechanical stretching on the expression of the *Tnc* and scleraxis gene (*Scx*) besides *col1* and *col3*, the markers for tendons and ligaments, has not been extensively studied. The present studies were, therefore, undertaken to investigate the effects of mechanical stretching on the cell morphology and the mRNA expression of critical genes regulating tendon/ligament genesis in rat MSCs (rMSCs), including *Col1*, *Col3*, *Tnc*, and *Scx*.

2 Materials and methods

2.1 rMSC isolation and cultivation

Sprague-Dawley rats (2–4 months) were housed in a specific-pathogen-free environment and fed a standard rodent diet. The animal experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. The femurs and tibias from the Sprague-Dawley rats were sawn and the gelatinous bone marrow was extracted under sterile conditions. The rMSCs were isolated by Percoll (1.073 g/mL) density centrifugation (Sigma-Aldrich, St. Louis, MO, USA) and subsequent adhesion to cell culture flasks. The cells were then cultured in Dulbecco's modified Eagle's medium (HyClone, Logan, UT, USA) supplemented with 10% fetal calf serum (HyClone, Logan, UT, USA) and 100 U/mL penicillin + 100 μg/mL streptomycin in a standard incubator under a humidified atmosphere of 5% CO_2/95% air at 37 ˚C. The culture medium was changed 2–3 times per week. After the cells reached 80–90% confluence (usually about 5–7 days), they were released with 0.25% trypsin/1 mM EDTA and subcultured at a density of 2.5 $\times$ 10^3 cells per cm^2. Nonadherent hematopoietic cells were removed with the medium when it was changed. Characterization of rMSCs was demonstrated as described previously (16). Cells from passages 2–5 were used for the experiments.

2.2 Application of mechanical stretching

To generate a stretching loading to cultured cells in vitro, we employed the mechanical stretching device (Model ST-140, STREX Co., Ltd, Osaka, Japan). The loading principle of this device is similar to an earlier published method (17). Briefly, it consists of a control unit, a strain

unit, and rectangular, elastic silicone chambers (as shown in Fig. 1A). During the stretching experiments, only the strain unit was put into the incubator. Simplified scheme of the device showing the principle of stretching was shown in Fig. 1B. The chambers were designed for use in the strain unit, which was driven by an eccentric motor that allowed variations in the magnitude and frequency of the applied strain. There were two types of chambers, designated ST-CH-04 and ST-CH-10. The ST-CH-04 chambers were 40 mm long, 25 mm wide, and 10 mm high, and the wells had a 20 mm $\times$ 20 mm cell culture surface. The ST-CH-10 chambers were 51 mm long, 35 mm wide, and 10 mm high, and the wells had a 32 mm $\times$ 32 mm cell culture surface. This automated instrument was designed to hold four ST-CH-04 or five ST-CH-10 chambers with precise uniaxial mechanical strain applied synchronously. The stretching over the entire cell culture surface is uniformity.

The cells were seeded at a density of 1×10^4 cells/cm^{2}into a chamber precoated with 5 μg/mL type I collagen (B&D Systems, Minneapolis, MN, USA) and allowed to reach nearly confluence after about two days cultivation. The chamber was then mounted on the strain unit of the strain instrument using four holes and hooks. One end of the chamber was firmly attached to a fixed frame and the other end was held on a movable frame. The movable frame was connected to a motor-driven shaft. The cells in the silicone chamber were exposed to stretching treatment with 0.1 Hz, 10% strain for 24 h or 48 h. As controls, static cells were cultured in a chamber under the same conditions, but were not subjected to any strain. For statistical analysis, the experiments were repeated at least three times.

2.3 Observation of cell morphology

After treatment with mechanical stretching, the changes in cell morphology and alignment of rMSCs were observed and photographed under an inverted phase-contrast microscope (Olympus, Japan).

2.4 RNA isolation, cDNA synthesis, and mRNA expression analysis of Col1, Col3, Tnc, and Scx by RT–PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions, and the RNA quality was assessed from the 18S/28S rRNA ratio. cDNA was synthesized in a reverse transcription reaction containing 2 μg of total RNA and reverse transcription reagents according to the manufacturer's instructions. Briefly, RNA was diluted first with 0.5 μg of oligo (dT) (Invitrogen, Karlsruhe, Germany) and 2 mM dNTPs (Invitrogen, Karlsruhe, Germany). The solution was heated to 70 $^\circ$C for 10 min in a thermocycler to dissolve the secondary RNA structures. The solution was then cooled to 4 $^\circ$C for 10 min and 2.4 μL of 25 mM $MgCl_2$, 2 μL of 10 $\times$ RT buffer (BioFlux, Tokyo, Japan), 2.6 μL of diethylpyrocarbonate (DEPC)-treated water, 5% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA), and 200 U of reverse transcriptase (BioFlux, Tokyo, Japan) were added. This solution was incubated at 42 $^\circ$C for 40 min, then the reverse transcriptase was inactivated by incubation at 94 $^\circ$C for 5 min. The sample was cooled to 4 $^\circ$C and stored for further analysis.

The primer sequences for RT–PCR are shown in Tab. 1. The synthesized cDNA was diluted 1:10 with DEPC-treated water. The cDNA (10 μL) was amplified in a solution containing 2 mM dNTPs, 1 μL each of the sense and antisense primers, 5 μL of 10 $\times$ PCR buffer (Bioflux, Tokyo, Japan), 10 μL of 5 $\times$ Q solution, and 2.5 U of *Taq* DNA polymerase (Takara, Shiga, Japan). PCR was performed using the Px2 Thermal Cycler (Thermo Hybaid, Ulm, Germany) and the PCR cycling parameters were 95 $^\circ$C for 3 min; 37 cycles of 94 $^\circ$C for 30 s, 55 $^\circ$C for 30 s, and 72 $^\circ$C for 55 s; followed by a final extension step at 72 $^\circ$C for 7 min, and cooling to 4 $^\circ$C. The amplified cDNA fragments were separated by electrophoresis on a 1.5% agarose gel (Bio-Rad, Foster City, CA, USA). The PCR products were visualized by staining with ethidium bromide (Takara, Shiga, Japan). The transcript expression of *Col1*, *Col3*, *Tnc*, and *Scx* was analyzed using the Gel Doc™XR Imaging System (Bio-Rad, Foster City,

Table 1: Oligonucleotide primer sequences used for RT–PCR analysis

Name	sequence 5′→3′	Product size	NCBI reference sequences (Ref Seq)
Type I collagen	Sense GAGCGATTACTACTGGATTGACCC Antisense CAAGGAATGGCAGGCGAGAT	506 bp	NM_053356
Type III collagen	Sense AAGAGCGGAGAATACTGGG Antisense CAATGTCATAGGGTGCGATA	532 bp	BC087039
Tenascin C	Sense AGATGCTACTCCAGACGGTTTC Antisense CACGGCTTATTCCATAGAGTTCA	200 bp	U15550
Scleraxis	Sense GACCGCACCAACAGCGTGAA Antisense GTGGACCCTCCTCCTTCTAACTTC	382 bp	S78079
$\beta-$actin	Sense CTGCCGCATCCTCTTCCTC Antisense CTCCTGCTTGCTGATCCACAT	398 bp	NM_031144

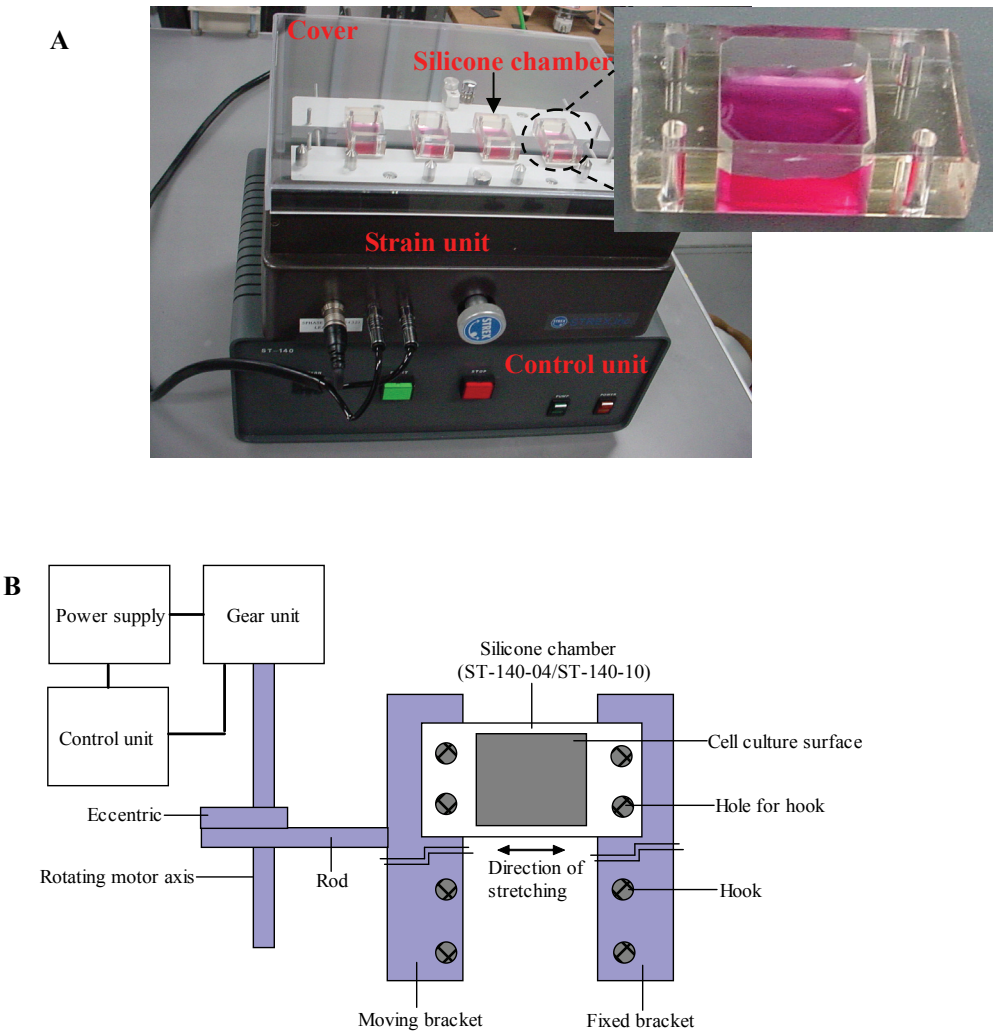

Figure 1: Mechanical stretching device for the application of strain. (A) Photograph of STREX Cell Stretching System (Model ST-140, Osaka, Japan). (B) Simplified scheme of the device showing the principle of stretching. The device consists of a control unit, a strain unit, and elastic silicone chambers. Cells are seeded on substrate in the chamber and the chamber is mounted on the strain unit using four holes and hooks. This device is designed to hold four ST-CH-04 or five ST-CH-10 chambers with precise uniaxial mechanical strain applied synchronously. During the stretching experiments, only the strain unit is put into the incubator.

CA, USA) and was normalized against the expression of the gene for β-actin (*Actb*).

2.5 Statistical analysis

The Data are expressed as means $\pm$ standard deviations from at least three separate experiments. Statistical analysis comparing the results of two groups was carried out with Student's t-test. A P value of less than 0.05 was considered to be statistically significant.

3 Results

3.1 Effects of mechanical stretching on cell morphology and alignment

rMSCs have an elongated fusiform shape resembling that of fibroblasts. The controls, cultured under static conditions without any strain, showed no particular orientation but were randomly oriented on the substrate (Fig. 2A, C). When the cells were subjected to 0.1 Hz, 10% strain for 24 h or 48 h, changes were induced in their morphology and particularly in their orientation. The stretched cells were oriented at approximately $\sim 65^{\circ}$ with respect to the stretching direction after stretch–elongation of the silicone chamber for 24 h (Fig. 2B). This alignment behavior was more obvious after 48 h of stretching (Fig. 2D). Moreover, the application of mechanical stretching promoted rMSCs with a more fibroblast-like cell shape (Fig. 2B, D). No cells detached from the substrate were observed during the experiments.

3.2 mRNA expression of Col1, Col3, Tnc, and Scx genes

The expression of *Col1*, *Col3*, *Tnc*, and *Scx* transcripts, normalized to that of the housekeeping gene *Actb*, was determined by RT–PCR. After 24 h of 0.1 Hz, 10% stretching, the expression of *Col1* and *Col3* was significantly higher than that in the control cells ($P < 0.01$ and $P < 0.001$, respectively). There was no detectable expression of *Tnc* or *Scx* in either the controls or the stretched cells at this point (Fig. 3 and Fig. 4A). When the cells were continuously subjected to the same stretching for 48 h, no significant differences were observed in the expression of *Col1* or *Col3* between the controls and the stretched cells. However, the expression of *Tnc* and *Scx*, the markers for tendons and ligaments, was detectable and more clearly observed in the stretched cells than in the controls, which showed no detectable expression of the *Tnc* or *Scx* gene at this point (Fig. 3 and Fig. 4B).

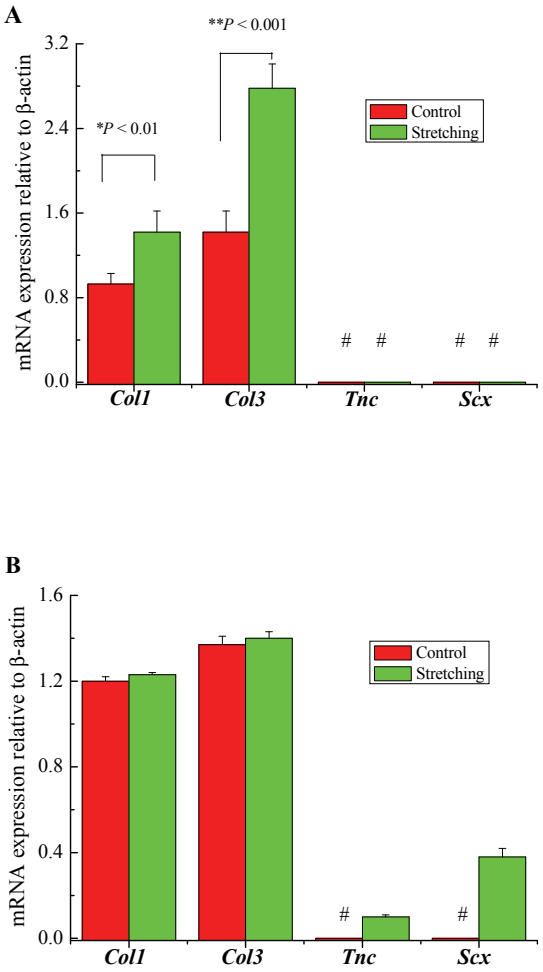

Figure 4: Relative mRNA expression of tendon/ligament-associated genes normalized to the expression of the housekeeping gene β-actin. A) Treatment of rMSCs with 0.1 Hz, 10% strain for 24 h; B) Treatment of rMSCs with 0.1 Hz, 10% strain for 48 h. Data represent means $\pm$ standard deviations of three separate experiments. *Col1*, type I collagen; *Col3*, type III collagen; *Tnc*, tenascin C; *Scx*, scleraxis. * $P < 0.01$; ** $P < 0.001$; # undetectable.

4 Discussion

Tissue engineering is a complex process involving the acquisition of autologous cells, cell isolation and cultivation, seeding on adequate scaffolds, and the engineering of a bioactive construct containing tissue-specific extracellular-matrix proteins (12). MSCs can act as the basis for the

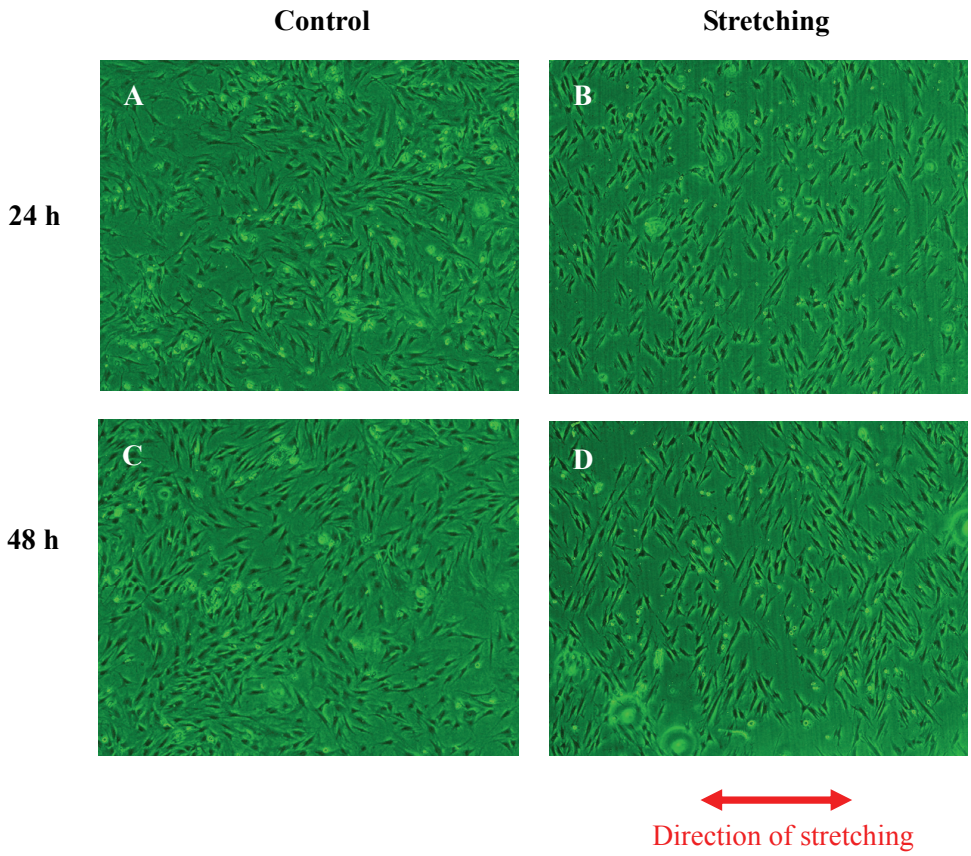

Figure 2: Effects of mechanical stretching on cell morphology and alignment of rat bone marrow-derived mesenchymal stem cells (rMSCs). rMSCs were seeded at a density of 1×10^4 cells/cm^{2}in a silicone chamber precoated with 5 μg/mL type I collagen, and subjected to mechanical stretching with 0.1 Hz, 10% strain for 24 h or 48 h. Control cells were randomly oriented at 24 h and 48 h (A, C). The stretched cells were oriented at approximately $\sim$65° with respect to the stretching direction and had a more fibroblast-like morphology (B, D). Original magnification: $\times$ 40. Arrowhead indicates the direction of stretching.

tissue engineering of autologous implants without concern for transplant rejection. They have been identified as an attractive cell source for various types of tissue engineering. In our previous research, we have proven that mechanical stretching plays an important role in regulating MSCs growth and proliferation (18). Titushkin et al. examined the biomechanical changes in hMSCs membrane and cytoskeleton in osteogenic differentiation, and demonstrated the important role of external physical force to regulate stem cell fate (19). A recent study by Kobayashi et al. described the effects of mechanical stress on the proliferation and differentiation of bone marrow-derived mesenchymal stem cells, through attachment interactions between the stem cells and scaffold matrix (20). In the present study, we explored the effects of mechanical stretching on rMSCs morphology and mRNA expression of tendon/ligament-associated genes, including *Col1*, *Col3*, *Tnc*, and *Scx*, which encode essential extracellular-matrix proteins and specific markers for tendon/ligament development.

Our results show that the application of mechanical stretching led to an obvious change in the cellular morphology and orientation of rMSCs. After exposure to 0.1 Hz, 10% strain for 48 h, most cells were oriented at approximately $\sim$65° with respect to the stretching direction and developed

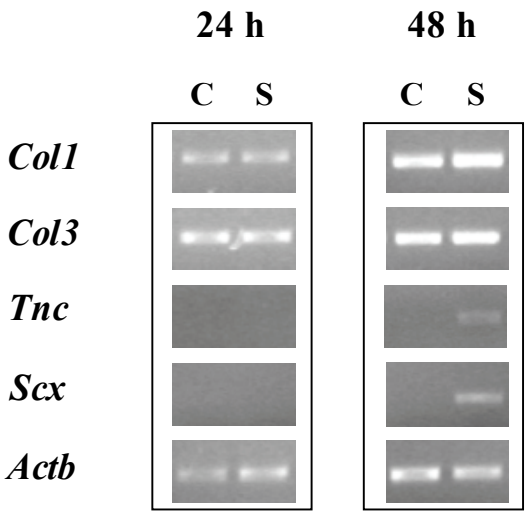

Figure 3: Representative 1.5% agarose gels indicating the mRNA expression of tendon/ligament-associated genes detected by RT–PCR in rMSCs treated with 0.1 Hz, 10% strain for 24 or 48 h. C, control; S, stretched; *Col1*, type I collagen; *Col3*, type III collagen; *Tnc*, tenascin C; *Scx*, scleraxis; *Actb*, β-actin.

a more fibroblast-like elongated phenotype (Fig. 2). These changes are consistent with the findings of Moretti et al., who reported a perpendicular reorientation of endothelial cells under cyclic mechanical stimulation (21), and of Koike et al., who described changes in the orientation of bone-marrow stromal ST2 cells after exposure to mechanical strain (22). Hayakawa et al. speculated that the stress fibers aligned along the stretching direction were torn soon after stretching and subsequently became reoriented obliquely to the direction of stretching (23). However, the details of the mechanism of stretch-dependent cell reorientation are still unclear.

At present, few genes have been reported as general markers that can be used to delineate tendon/ligament development. Despite their unique architecture, most structural components of tendons/ligaments are also expressed in other connective tissues (24, 25). Nöth et al. found that a collagen type I hydrogel concentration of 3.0 mg/mL in combination with cyclic strain (frequency: 1 Hz, amplitude: 3 mm) can lead to anterior cruciate ligament constructs fabricated from human MSCs, which based on the analysis of gene expression of collagen type I and III, fibronection and elastin (26). However, those four genes are not specific to tendon/ligament fibroblasts. Tenascin has been used as a tendon marker, but it is also expressed in cartilage and nerves (27). Recently, scleraxis has been reported to be a unique marker of tendon/ligament progenitor cells (10, 11), and provides a genetic tool to support broad research into tendon/ligament genesis and patterning. Although some previous studies have evaluated the effects of chemical or mechanical stimulation (12, 13, 14) or their combined effects (26, 28) on the promotion of gene expression for the purpose of tendon/ligament engineering, we have found no analysis of the *Col1*, *Col3*, *Tnc*, and *Scx*, the several key genes modulating tendon/ligament genesis, in these studies. In this study, we explored the effects of mechanical stretching on the mRNA expression of these critical tendon/ligament-associated genes, including *Col1*, *Col3*, *Tnc*, and *Scx*. The stretch parameter (0.1 Hz, 10% strain) used in our study were chosen in accordance with previous investigations, which implied that these strain values favor cell differentiation (4, 6, 29, 30). Our data reveal that 0.1 Hz, 10% stretching for 24 h caused the profound upregulation of the mRNA expression of *Col1* and *Col3* in rMSCs, indicating that the stretched cells assume a synthetic phenotype.

More importantly, 0.1 Hz, 10% stretching for 48 h induced clear mRNA expression of *Tnc* and *Scx*, the markers for tendons and ligaments. In contrast, the controls exhibited no detectable mRNA expression of *Tnc* and *Scx*. This result is consistent with the findings of other investigators regarding the expression of *Col1*, *Col3*, and *Tnc* (13, 26). Together with the results for *Scx* expression in our study, it suggests that the mechanical stretching could induce the directed differentiation of rMSCs into tendon/ligament fibroblasts in vitro.

It is necessary to emphasize that *Col1*, *Col3* and *Tnc* are not fibroblast specific. Bone and connective tissues are rich in *Col1* and *Col3*, and *Tnc* is also found in cartilage and nerves. However, *Col1* and *Col3* are often chosen as the primary reference proteins because they are the predominant constituents of native tendon/ligament tissue (31). *Tnc*, an extracellular protein, is an excellent marker for tendon development, has been used extensively to detect early tendon primordia, and is also found within differentiated tendons (27, 32). In this study, besides *Col1*, *Col3*, and *Tnc,* we analyzed the cell fate using *Scx*, the early specific marker for tendons and ligaments. Consequently, the capacity to induce the upregulation of *Col1* and *Col3* expression and the detectable expression of *Tnc* and *Scx* can be regarded as a fundamental step in driving rMSCs towards a fibroblast lineage and directed tendon/ligament development.

In conclusion, in this study, we have demonstrated that rMSCs, when subjected to 0.1 Hz, 10% stretching, exhibit obvious detectable mRNA expression of *Tnc* and *Scx*, and a significant increase in the expression of *Col1* and *Col3*. The stretched cells orient at approximately $\sim 65^{\circ}$ with respect to the stretching direction and exhibit a more fibroblast-like cell type. These findings suggest that mechanical stretching could induce the directed differentiation of rMSCs toward a tendon/ligament fibroblast lineage in vitro, which may benefit wound healing in injured tendons/ligaments and the development of a tissue-engineered tendon/ligament construct.

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